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(71) Applicants
Jouko Juhani Kankare
Paljaspaa 8 E 3, SF-206 10 Turku 61, Finland

Keijo Ensio Haapakka
Turku, Finland

(72) Inventors
Jouko Juhani Kankare
Keijo Ensio Haapakka

(74) Agent and/or Address for Service
Mathys and Squire
10 Fleet Street, London, EC4Y 1AY, United Kingdom

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(58) Field of search
UK CL (Edition J) G1A ACJ, G1B BBC
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(54) Electrochemical analysis of analytes in solution

(57) A method whereby the presence and/or amount of a chemical moiety containing lanthanide, e.g. terbium or europium is determined by applying an electrical pulse into an electrode immersed in a solution and measuring the delayed light emission after some time from the end of the pulse, said chemical moiety being either bound to said electrode and/or present in said solution and said emitted light being taken as an indication of the amount of the chemical moiety present in the proximity of said electrode. The method is useful especially for analytical methods based on binding assays such as immunoassays and nucleic acid hybridization assays.

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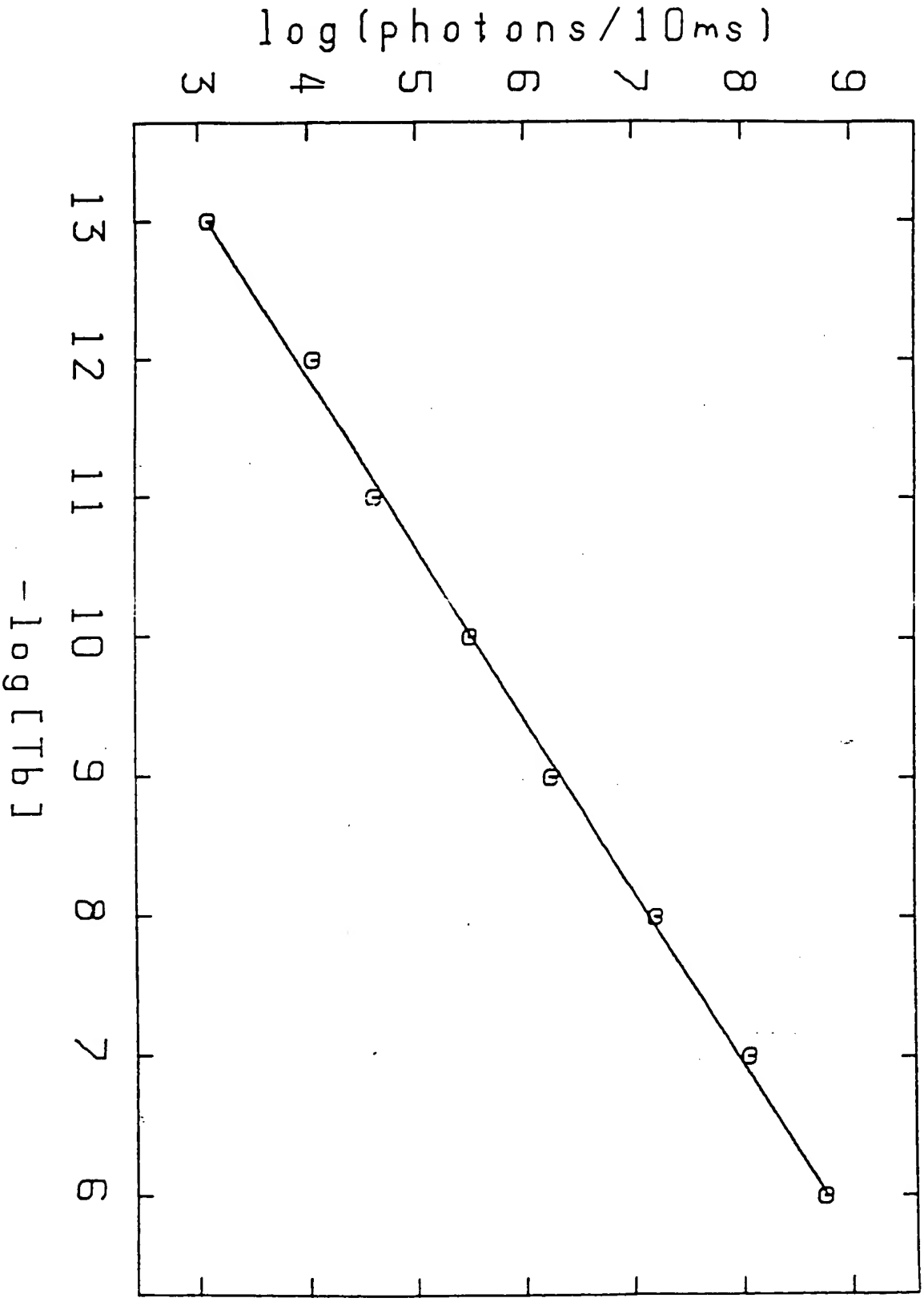


FIG. 1

Fig. 2.

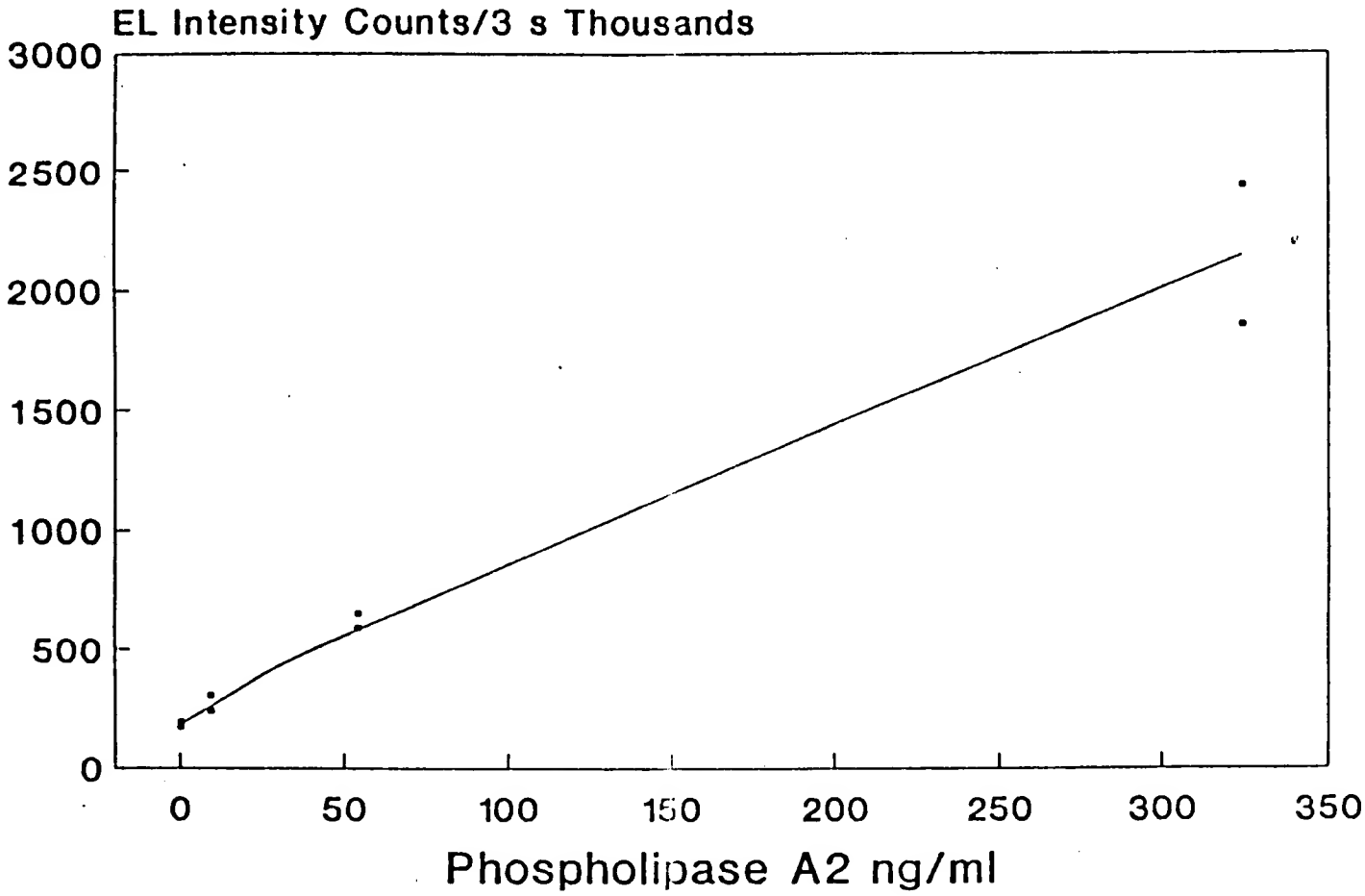
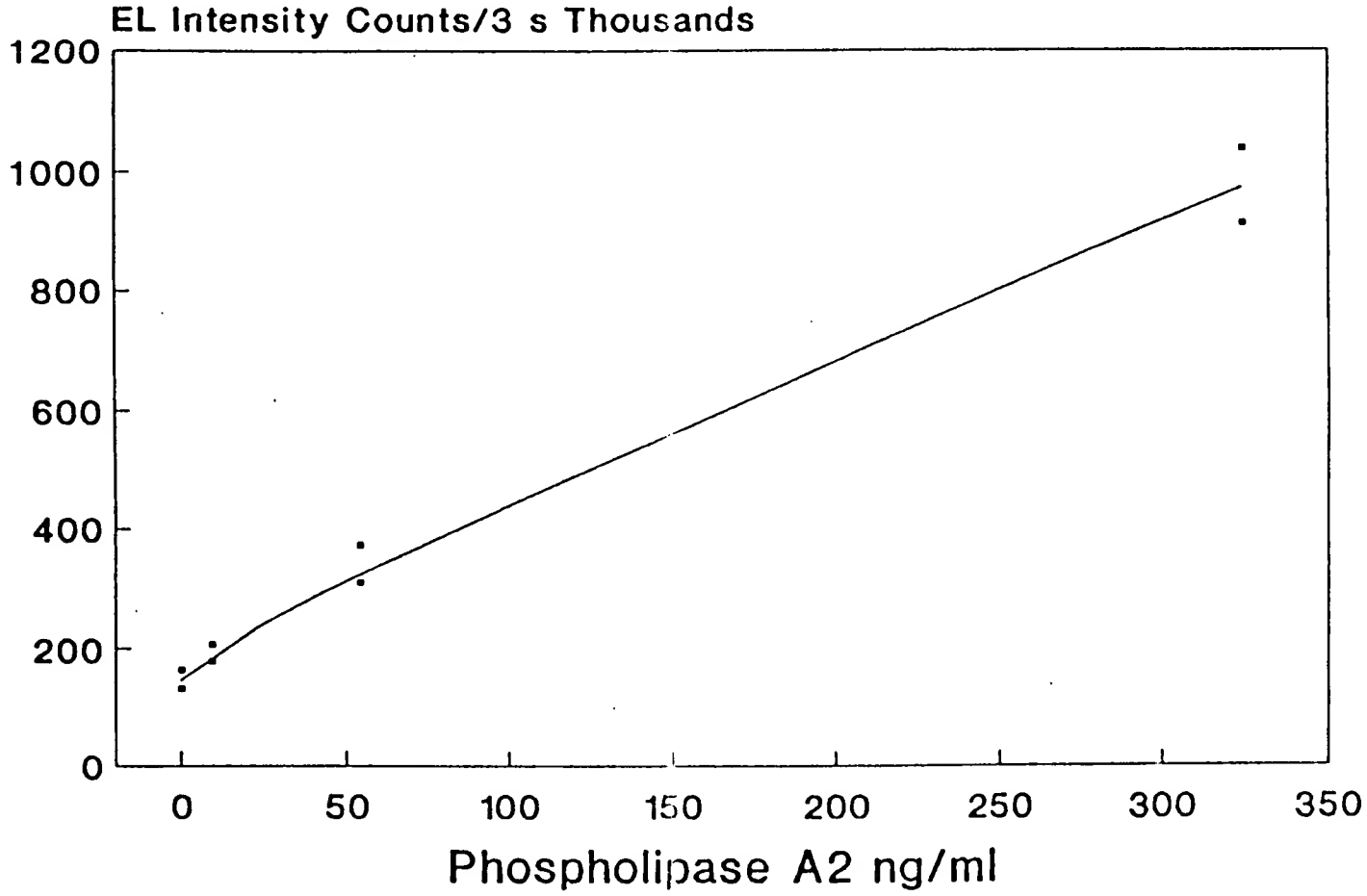

EL Intensity Counts/3 s Thousands
3000
2500
2000
1500
1000
500
0
0
50
100
150
200
250
300
350
Phospholipase A2 ng/ml

Fig. 3.



ELECTROGENERATED LUMINESCENCE IN SOLUTION

Field of Invention

The invention pertains to the method whereby a luminescent compound in aqueous or nonaqueous solution is excited by an electrical pulse either directly by electron transfer from an electrode or indirectly by some mediating electrochemically induced reaction. The light emission from the compound is detected after the end of the excitation pulse.

The new method may find applications in those fields where a very low detection limit is required, e.g. in the analytical methods based on binding assays such as immunoassays and nucleic acid hybridization assays.

The analytical methods based on luminescence in its various modifications are generally known for their sensitivity, but each have their shortcomings at very low concentrations of the emitting species. The sensitivity of fluorescence is limited by Rayleigh and Raman scattering phenomena as well as fluorescent impurities which increase the nonspecific background emission. Phosphoreecence is mainly restricted to solid state and the emission of those very few compounds which have room temperature phosphorescence in solution is generally extremely sensitive to oxygen, which hampers their practical applications. The delayed fluorescence of some lanthanide chelates has been used as a basis of an immunoassay method, and it allows a very low detection limit. The methods based on the conventional fluoreescence and phosphorescence use excitation by light and need appropriate light source and optics. The methods based on chemiluminescence (CL) do not need excitation optics and the instrumentation is generally very simple. However, the CL methods are often subject to serious chemical interf rence.

The method suggested in this inv ntion circumvents certain shortcomings of other lumin scence methods. No excitation optics are needed and the electronic instrumentation required for the pulse excitation by electric current can be made very simple. The essence of the invention is that the nonspecific background emission is totally eliminated by using appropriate luminescent compounds with long-lived luminescence and by measuring light emission after some time delay from the end of the excitation pulse.

Description of the Prior Art

Electrogenerated chemiluminescence (ECL) has been known for a long time. Its use in immunoassay has been proposed by Bard et al. (D. Ege, W. Becker and A. Bard, Anal. Chem. 56 (1984) 2413, PCT Int. Appl. WO 86/02734). They suggest to use ruthenium- or osmium-containing compounds as labels in binding assay. Platinum and glassy carbon are used as the material for the working electrode in the example given, and the light emission from the electrode is measured during the potential pulse.

As shown by the present authors, electrogenerated luminescence is generated at oxide-covered aluminum or tantalum electrodes by numerous inorganic ions (K. Haapakka et al., Anal. Chim. Acta 171 (1985) 259) and fluorescent organic compounds (K. Haapakka et al., Anal. Chim. Acta 207 (1988) 195) in the presence of suitable oxidizing agents. In these studies the light emission from the electrode was measured also during the potential pulse applied the electrodes.

It would be very advantageous to have a method which allows inexpensive, preferably disposable electrodes and makes use of compounds having long-lived luminescence which is relatively free of interferences. Such a method would find use e.g. in binding assays such as homogeneous and heterogen ous immunoassay allowing rather simple and inexpensive instrumen-

tation. In the immunoassay or more generally binding assay two components react specifically with each other and the product is quantitated by a suitable, highly sensitive method. If it is necessary to separate the product before its determination, the method is called heterogeneous, and homogeneous if no separation step is necessary. Because of a simpler procedure, homogeneous assays are preferable, but so far heterogeneous assays have provided lower detection limits. Typically in these methods the presence of a compound is indicated by labelling it with a chemical moiety which can be determined with a high sensitivity, e.g radioactive isotope, enzyme, fluorescent compound, etc. Especially advantageous is labelling with a fluorescent compound which has a slow emissive decay of the excited state. Most of the samples subjected to immunoassay contain natural fluorescent species which increase the background emission and consequently impair the detection limit in the conventional fluorometric determination. Chelates of europium and terbium have the lifetime of their fluorescence emission in the millisecond region, i.e. several orders of magnitude longer than the "natural" fluorescence of organic compounds of biological origin.

Homogeneous assay based on luminescence is possible if the antibody-antigen complex adsorbed on the surface can be selectively excited without the excitation of labelled compound in the solution. This has been previously achieved (U. S. Pat. 3,939,350 (1976)) by using labelled antigens bound to antibodies linked to a quartz slide. The sample is excited from another side of the slide, with the beam totally reflecting from the slide surface. In the measurement only the solid-phase bound fraction is excited, thus obviating the separation step. The method places strict optical quality requirements on the sample slide and consequently its use in routine assays is restricted. Also the scattering and background fluorescence remain severe problems. Preferential excitation of luminescent compounds on the surface or its immediate vicinity can be technically more easily achieved by

using electrogenerat d luminesc nce and influence of background flucrescence can be minimized by using labels with delayed electroluminescence.

Detailed Description of the Invention

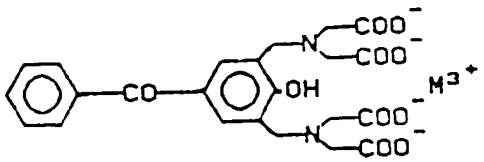
The invention pertains to a method for the determination of the presence and/or amount of a chemical moiety containing terbium or europium by applying an electrical pulse into an electrode immersed in a solution containing the said moiety as a solute and/or adsorbed onto the surface of the electrode, and measuring the delayed light emission after some time from the end of the pulse. The measured light emission is taken as an indication of the amount of the chemical moiety present in the proximity of the electrode. The phenomenon to be measured will be called here delayed electroluminescence or DEL for brevity.

The said chemical moiety may have a general structure

$$(M\text{-}Z)_n\text{-}L_m\text{-}Y_p$$

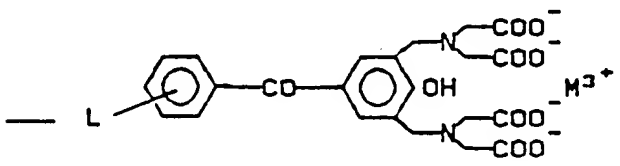
wherein M is terbium or europium, n is an integer greater or equal to one, m and p integers equal to or greater than zero, Z is a polydentate ligand, L is a linking group and Y is a substance to be described later. Z, L and Y are of such composition that the chemical moiety can be induced to emit light by subjecting it to the conditions required by the delayed electroluminescence.

In the simplest case m and p are both zero and n is one. In this case M-Z is a chelate of terbium or europium. One preferred structure of M-Z is



$M^{3+} = Tb^{3+}$ or Eu^{3+}

The method can be used .g. for the highly sensitive determination of terbium as will be described in Example I.
In a more complicated case n,m≥1 and p≥1. Substance Y is then said to be labelled by a DEL label. Suitable substances Y include many biological substances, e.g. whole cells, subcellular particles, viruses, nucleic acids, nucleotides, oligonucleotides, polynucleotides, polysaccharides, proteins, polypeptides, enzymes, cellular metabolites, hormones, pharmacological agents, alkaloids, steroids, vitamins, amino acids, carbohydrates, serum-derived or monoclonal antibodies. It is within the scope of this invention to include also synthetic substances, such as drugs, synthetic nucleic acids and synthetic polypeptides. Substance Y is linked through linking groups L to chelates M-Z. The linking groups may be those bivalent radicals generally used for labelling analyte molecules by probe molecules well known to those of ordinary skill in the art. These bivalent linking groups include a ureido, thioureido, an amide, such as -CONH-, -CONMe-; thioether, such as -S-, -S-S-; sulfonamide, such as $-SO_2NH-$, $-SO_2NMe-$. Linking group L may also contain a molecular chain of variable length and composition called spacer. This spacer is used to keep the chelate part and substance Y at a suitable distance from each other and it may have aforementioned bivalent linking groups as sidegroups. One part of these sidegroups are bound to the polydentate ligands Z, another part to substances Y. Polydentate ligand Z may be an aromatic compound having chelating sidegroups such as $-CH_2N(CH_2COOH)_2$. One preferred structure of the chelate is:



M^{3+} = Tb^{3+} or Eu^{3+}

The invention may be used to determine labelled moieties of interest, to employ labelled moieties to determine analytes of interest, or to use labelled analogues of analytes of interest

to determin analytes of interest in both competitive and noncompetitive binding assays. These binding assays may be heterogeneous or homogeneous. Analogous binding assays are used also in nucleic acid hybridization techniques, where the DEL labels could also find use, such as dot-blot and sandwich hybridization assays as well as hybridization assays employing affinity based collection and PCR (polymerase chain reaction) technology.

For instance, in a competitive immunoassay antibody is coated onto the electrode surface and antigen and antigen with a DEL label compete for the active sites of the antibody. Antigen now corresponds to substance Y and may belong to one of types described previously. The amount of antibody-antigen complex on the electrode surface is quantitated by DEL either directly after immunoreaction or after a washing step and addition of suitable electrolyte solution containing e.g. peroxydisulfate. Alternatively homogeneous noncompetitive immunoassay can be achieved by immobilizing a "catching" antibody on the electrode surface. The sample antigens caught by those antibodies are quantitated with use of DEL labelled antibodies that bind to a second site on the antigen. In this case antigens may be substances listed previously in connection with the definition of Y.

A. Apparatus

No patent claims about the apparatus are made but a detailed description of it is given in order to give a clearer picture on the method of measurement.

The measuring system is composed of a pulse generator, potentiostat, a sample cell with two or three electrodes, an

optional light filter or monochromator, a light detector and a gated integrator or photon counter. The pulse generator may be any generator which is capable of producing freely program-

mable pul e chains with adjustable amplitude.

The potentiostat may be a conventional three-electrode potentiostat, or, if only two electrodes are used, a simple booster amplifier capable of delivering a few tens of milliamperes of current.

The sample cell and light detector are enclosed in the same light-tight chamber. The cell has two or three electrodes immersed into the electrolyte solution. In case of three electrodes one electrode is a reference electrode, one is an auxiliary electrode and one is a working electrode. These are connected to the potentiostat by the conventional way. Light emission is measured from the working electrode, which is made of any conducting material. Preferable material is oxide-covered metal, e.g. aluminum, tantalum, zirconium or hafnium The reference electrode may be any conventional reference electrode, e.g. calomel electrode or Ag-AgCl electrode. The auxiliary electrode may be made of any conductive material, most often platinum. If only two electrodes are used the electrodes can be both made of the same material, e.g. aluminum, in which case light can be made to emit from both electrodes or the other electrode is made of different material. Alternatively the sample cup itself may be made of aluminum and it functions in this case as the working electrode from which light is emitted.

The light intensity from the working electrode is measured using a photomultiplier or a photodiode with an optional filter or monochromator in between, and the electrical signal from the light detector is brought to a gated integrator or a gated photon counter. Gating is synchronized with the pulses from the pulse generator with an appropriate delay.

B. Method

The sample to be measured for its DEL is a compound which is dissolved in solution or adsorbed onto the surface of the working electrode. The compound should have a slow decay of its electroluminescence. Preferred compounds are luminescent lanthanide complexes, preferably such as chelates of Tb^{3+} or Eu^{3+}, which have their decay at the millisecond timescale. The compound may be measured itself or it may be bound as a label to the material to be assayed. In addition to the compound to be measured the electrolyte solution in the sample cell contains some electrolyte, preferably sulfate or acetate to increase the conductivity. An oxidizing compound, such as peroxydisulfate, hydrogen peroxide or dissolved oxygen may be present in the solution. The function of the oxidizing agent is to produce highly reactive radicals by a direct or mediated electrolytic reduction,

e.g. $S_2O_8^{2-} + e^- \rightarrow SO_4^{2-} + SO_4^{\cdot}$

These radicals react with the luminescent compound producing light emission. Consequently electroluminescence is observed after a cathodic pulse to the working electrode. Potentially anodic pulses may be used for certain types of lanthanide compounds.

A sequence of cathodic pulses with a suitable duration and duty cycle depending on the luminescent compound is applied to the working electrode. The resulting light emission is measured after the end of the cathodic pulses using an appropriate gate width and delay. For the preferred terbium complexes the length of the cathodic pulse may vary from 0.2 ms to 5 ms, the delay after the pulse is 0.1 to 0.5 ms and the gate width from 2 ms to 10 ms. For the europium complexes the times are ca. 4 times shorter. The signal integrated during the open gate time is averaged for as many periods as is necessary to achieve the required signal-to-noise ratio.

EXAMPLE I

Standard curve for terbium by electroluminescence

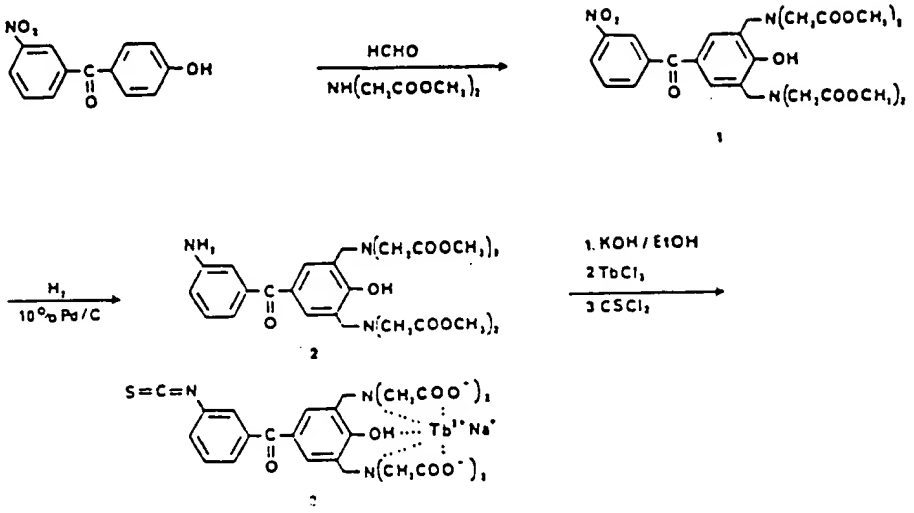
The sample solution in the example is 0.3 M in sodium sulfate, 0.001 M in potassium peroxydisulfate and 10^{-5} M in 2,6-bis-[N, N-bis(carboxymethyl)aminomethyl]-4-benzoylphenol, and adjusted to pH 11.2 with 5×10^{-4} M TRIS and NaOH. The DEL measurements were done in disposable cups made of aluminum sheet of 0.3 mm thickness and 99.9% purity. The other electrode was a short platinum wire. Increasing portions of terbium chloride were added and the delayed electroluminescence was measured by using cathodic pulses of 1 ms duration, 8.5 V amplitude and 4% duty cycle. The light emitted from the aluminum cup was detected by a photomultiplier and a two-channel photon counter (Stanford Research, Model SR400). The gate of one channel was open from 0.2 to 10 ms from the end of the cathodic pulse and the other channel counted the "dark current" photons from 10.2 to 20 ms. After 100 s counting time the contents of the two counter registers were subtracted from each other. Table 1 and Fig. 1 show the results.

TABLE I

Terbium mol/L	Photons/100 s
10^{-13}	1,200
10^{-12}	11,000
10^{-11}	40,800
10^{-10}	316,000
10^{-9}	1,750,000
10^{-8}	15,824,000
10^{-7}	112,000,000
10^{-6}	565,000.000

EXAMPLE II

Preparation of a labelling compound



Scheme.

Synthesis of 4-(3-nitrobenzoyl)-2,6-bis[N,N-bis(methoxycarbonylmethyl)aminomethyl]phenol (1).

To a solution of 37% aqueous formaldehyde (0.81 g, 10 mmol) in methanol (20 mL) was added dimethyl iminodiacetate (1.61 g, 10 mmol). The solution was concentrated in vacuo. Another portion of methanol (25 mL) was added to the residue and the solution was concentrated in vacuo. To the remainder 4-hydroxy-3'-nitrobenzophenone (1.22 g, 5 mmol) was added, and the mixture was heated with stirring at 110 °C for 20 h. The product was purified by chromatography on silica gel using chloroform as the eluent. The yield of yellowish oil was 1.76 g (60%). ^{1}H NMR ($CDCl_3$): δ 3.48 (1H, s), 3.58 (8H, s), 3.71 (12H, s), 4.08 (4H, s), 7.73 (2H, s), 7.56-8.58 (4H, m).

Synthesis of 4-(3-aminobenzoyl)-2,6-bis[N,N-bis(methoxycarbonylmethyl)aminomethyl]phenol (2).

Compound 1 (0.89 g, 1.5 mmol) was stirred for 1 h in methanol (50 mL) with 10% Pd/C (90 mg) under hydrogen pressure of 50 psi. The mixture was filtered and evaporated in vacuo. The product was purified by chromatography on silica gel using light petroleum (b.p. 50-70 °C)/ethyl acetate (2:5) as the eluent. The yield of yellowish oil was 0.40 g (48%). ^{1}H NMR ($CDCl_3$): k 3.56 (1H, s), 3.59 (8H, s), 3.71 (12H, s), 4.01 (6H, broad s), 7.05-7.14 (4H, m), 7.70 (2H, s).

Synthesis of terbium complex of 4-(3-isothiocyanatobenzoyl)-2,6-bis[N,N-bis(carboxymethyl)aminomethyl]phenol (3).

Compound 2 (0.40 g, 0.71 mmol) was stirred for 3 h in 0.5 M KOH-ethanol (20 mL) and water (5 mL). The mixture was neutralized with 1 M HCl and evaporated in vacuo. Water (15 mL) and terbium chloride (0.27 g, 0.72 mmol) were added, pH was adjusted to 8.0 and the mixture was filtered. A few milliliters of acetone was added to the filtrate, and the terbium complex was filtered off. A small portion of the complex (68 mg) in water (3 mL) was added dropwise into a mixture of thiophosgene (31 μL, 0.4 mmol) and $NaHCO_3$ (42 mg, 0.5 mmol) in $CHCl_3$. After stirring for 1 h the water layer was separated and washed with $CHCl_3$. After adding a few milliliters of acetone the precipitate was filtered off and purified by chromatography on silica gel using CH_3CN/H_2O (4:1) as the eluent. The yield was 15 mg (38%; based on 2).

EXAMPLE III

Heterogeneous sandwich immunoassay of human pancreatic phospholipase A2.

Labelling of sheep-anti-human PLA_2 antiserum:

A 60-fold molar excess of 4-(3-Isothiocyanatobenzoyl)-2,6-bis[N,N-bis (carboxymethyl)-aminomethyl]phenol terbium complex (3, Example II) was allowed to react with the antibody at pH 9.5 overnight. The labelled antibody was separated from excess free terbium complex on a column filled with Sephadex G-50 (1x5.5 cm) and Sepharose 6 B (1x5.2 cm) by using 0.1 M sodium carbonate buffer pH 9.3, containing 9 g/L of NaCl and 0.05 % NaN_3 as the eluting agent.

Coating of the aluminum cups:

The aluminum cups (made of 99.9% aluminum foil of 0.3 mm thickness) were coated with anti-human PLA_2 antiserum by physical adsorption in 0.05 M Tris-HCl buffer, pH 7.5, containing 9 g/L of NaCl and 0.05% NaN_3 (TSA-buffer) for overnight at room temperature. After coating the cups were washed with a wash solution (NaCl 9g/L, NaN_3 0.01 % and Tween 20 0.2 g/L) and saturated with 0.1% bovine serum albumin (BSA) for overnight and stored wet at +4 C.

Immunoassay:

The aluminum cups were washed once with 500 µL of wash solution. Then 25 µL of standards containing 0, 9, 54 and 324 ng/mL of phospholipase A_2 in TSA-buffer (0.1 % BSA) were added to the cups followed by 175 µL of Tb-labelled anti-PLA_2 antibody (570 ng/mL) in 0.05 M Tris-H_2SO_4 buffer, pH 7.8, containing BSA 5 g/L, NaN_3 0.5 g/L. After incubation for 3 h by continuously shaking the cups were washed 6 times with the wash solution. The electroluminescence was measured in the cups after adding 450 µL of 0.001 M Tris-H_2SO_4 buffer, pH 8.7, containing 0.3 mol/L Na_2SO_4 and 0.001 mol/L $K_2S_2O_8$, as in Example I except that the counting time was only 3 s. The results of the assay are shown in Table 2 and Fig. 2.

TABLE 2

PLA_2 ng/mL	Photons/10^5/3s	
0	1.9	1.7
9	3.0	2.4
54	6.5	5.9
324	18.6	24.4

EXAMPLE IV

Homogeneous sandwich immunoassay of human pancreatic PLA_2 in serum.

Coating of the cups and labelling of sheep-anti-human PLA_2 antiserum were done as in Example III.

Immunoassay:

The aluminum cups were washed once with 500 µL of wash solution. Then 25 µL of standards containing 0, 9, 54 and 324 ng/mL of phospholipase A_2 in human serum were added to the cups followed by 425 µL of Tb-labelled anti-PLA_2 antibody (235 ng/mL) in 0.05 M Tris-H_2SO_4 buffer, pH 8.7, containing BSA 5 g/L, NaN_3 0.5 g/L, 0.3 mol/L Na_2SO_4, 0.001 mol/L $K_2S_2O_8$. After incubation for 3 h by continuously shaking the electroluminescence was measured directly in the cups as in Example I except that the counting time was only 3 s. The results of the assay are shown in Table 3 and Fig. 3.

TABLE 3

PLA_2 ng/mL	Photons/10^5/3s	
0	1.6	1.5
9	1.8	2.1
54	3.1	3.7
324	9.1	10.4

CLAIMS

1. A method whereby the presence and/or amount of a chemical moiety containing lanthanide, preferably terbium or europium, is determined by applying an electrical pulse into an electrode immersed in a solution and measuring the delayed light emission after some time from the end of the pulse, said chemical moiety being either bound to said electrode and/or present in said solution and said emitted light being taken as an indication of the amount of the chemical moiety present in the proximity of said electrode.

2. A method according to claim 1, wherein the said chemical moiety has the formula:

$$(M - Z)_n - L_m - Y_p$$

wherein:

M is terbium or europium;
Z is a polydentate ligand of M;
L is a linking group, such as a ureido, thioureido, an amide, such as -CONH-, -CONMe-; thicether, such as -S-, -S-S-; sulfonamide, such as $-SO_2NH-$, $-SO_2NMe-$; L may also contain a molecular chain of variable composition and length, which is bound to the polydentate ligands Z through one part of aforementioned bivalent groups and to substances Y by the other part;
Y is a substance attached to Z through one or more linking groups L;
n is an integer equal to or greater than 1;
p is an integer equal to or greater than zero;
m is an integer equal to or greater than zero;

3. A method according to claim 1 or claim 2, wherein said moiety is capable of binding to a chemical agent.

4. A competitive binding method of determining the presence

of an analyte of interest wherein the analyte and a chemical moiety bind competitively to a chemical material, the chemical moiety having the formula: $(M - Z)_n - L_m - Y_p$

wherein:

M is a lanthanide, preferably terbium or europium;

Z is a polydentate ligand of M;

L is a linking group, such as a ureido, thioureido, an amide, such as -CONH-, -CONMe-; thioether, such as -S-, -S-S-; sulfonamide, such as $-SO_2NH-$, $-SO_2NMe-$; L may also contain a molecular chain of variable composition and length, which is bound to the polydentate ligands Z through one part of aforementioned bivalent groups and to substances Y by the other part;

Y is a substance attached to Z through one or more linking groups L ;

n is an integer equal to or greater than 1;

p is an integer equal to or greater than 1;

m is an integer equal to or greater than 1;

the method comprising:

a) contacting the material, the chemical moiety and the analyte under suitable conditions so as to form a reagent mixture;

b) inducing the chemical moiety to emit light by applying an electric pulse to an electrode immersed into the reagent mixture;

c) detecting the emitted light after some time delay from the electrical pulse and thereby determining the analyte of interest.

5. A method according to claim 2, 3 or 4, wherein Y is a whole cell, subcellular particle, virus, nucleic acid, polysaccharide, protein, polypeptide, enzyme, cellular metabolite, hormone, pharmacological agent, drug, alkaloid, steroid, vitamin, amino acid or carbohydrate.

6. A method according to claim 2, wherein Y is a nucleotid , oligonucl otide or polynucleotide.

7. A method according to claim 5, wherein Y is an antibody.

8. A method according to claim 3, wherein said chemical agent is a whole cell, subcellular particle, virus, nucleic acid, polysaccharide, protein, polypeptide, enzyme, cellular metabolite, hormone, pharmacological agent, drug, alkaloid, steroid, vitamin, amino acid or carbohydrate.

9. A method according to claim 3, wherein said chemical agent is immobilized on the surface of at least one of the electrodes.

10. A method according to claim 3, wherein said chemical agent is an antibody.

11. A method according to claim 4, wherein said analyte is a whole cell, subcellular particle, virus, nucleic acid, nucleotide, oligonucleotide, polynucleotide, polysaccharide, protein, polypeptide, enzyme, cellular metabolite, hormone, pharmacological agent, drug, alkaloid, steroid, vitamin, amino acid or carbohydrate.

12. A method according to claim 11, wherein said analyte is an antibody.

13. A method according to claim 4, wherein said chemical material is an antibody.

14. A method according to claim 3, wherein said chemical agent is an analyte specific antibody immobilized on the surface of the electrode, Y is an antibody against a different or identical epitope of the analyte, and the analyte is attached between th antibodies, the method being a noncompetitive assay.

15. A method according to claim 14, wherein said analyte is a whole cell, subcellular particle, virus, nucleic acid, oligonucleotide, polynucleotide, polysaccharide, protein, polypeptide, enzyme, cellular metabolite, hormone, pharmacological agent, alkaloid, or carbohydrate.

16. A method according to claim 3, 4, 14 or 15, wherein the method is a homogeneous method and the suitable conditions are such that bound chemical moiety and unbound chemical moiety are not separated before the emitted light due to the electrical pulses is detected.

17. A method according to claim 3, 4, 14 or 15, wherein the method is a heterogeneous method and the suitable conditions comprise a separation of bound chemical moiety and unbound chemical moiety prior to the application of electrical pulse to the electrode and measurement of the emitted light.

18. A method according to claim 1, wherein at least one of the electrodes is made of aluminium.

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